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54 Turning on of cytotoxicity.

57 The invention provides a method of turning on the cytolytic effector function of human cytolytic T-cells or human natural killer cells, the method involving contacting the cells with a substance or mixture of substances characterized in that the substance or the mixtures binds specifically to the T11 sheep erythrocyte binding glycoprotein of the cells and is capable upon the binding of turning on the cytolytic effector function. Also provided is a cDNA sequence encoding human T11 or a fragment thereof which is capable of inhibiting T-lymphocyte activation.

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5 Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Fig. 1 is a histogram showing lysis of B-lymphoblastoid target cell lines by T-cells treated with various monoclonal antibodies ( $\alpha$ T11);

Fig. 2 is a histogram showing the effect of anti-T11 antibodies on the cytolytic effector function of 10 various cell lines:

Fig. 3 is a histogram showing lysis of various target cells by T-cell clones QQ and JT3:

Fig. 4 is a diagrammatic representation of the strategy used in sequencing the cDNA (PB1) encoding

Fig. 5 is the sequence of the PB1 cDNA, with the deduced amino acid sequence of T11 given on the bottom line; and

**Fig. 6 is a diagrammatic representation of the T11 molecule, showing functional domains.**

### Purification and Characterization of T11

4 × 10<sup>10</sup> Jurkat cells were washed twice in serum-free medium and lysed for one hour at 4° C in 420 ml in 10mM Tris, pH 8.0 containing 0.15 M NaCl, 1% Triton-X 100, 1 mM iodoacetamide and the following protease inhibitors (Sigma): phenylmethylsulfonylfluoride (1mM), chymostatin (0.5 µg/ml), pepstatin (0.5 µg/ml), antipain (0.5 µg/ml), leupeptin (0.5 µg/ml), trypsin inhibitor (0.02 µg/ml). The crude lysate was centrifuged at 3000 × g for 20 min. The supernatant was made 0.5% in sodium deoxycholate and ultracentrifuged for 60 min at 150,000 × g. 25 × 10<sup>6</sup> Jurkat cells from the same culture were surface radiolabelled by lactoperoxidase-catalyzed iodination. 2 × 10<sup>7</sup> radiolabelled cells were treated with 0.5 ml of lysis buffer and added to the large scale lysate. The combined lysates were applied at 0.75 ml/min to a 15 ml pre-clear column containing irrelevant mouse monoclonal antibodies anti-T3 (8C8), anti-T<sub>1</sub> (9H5) and anti-β2 microglobulin coupled to protein A Sepharose beads CL-4B (Pharmacia) at 5 mg antibody per ml of beads, followed by a 5 ml specific antibody column containing anti-T11<sub>1</sub> (8B5) coupled to protein A Sepharose at 5 mg/ml. The anti-T11 column was washed with 10mM Tris, pH 8.0 with or without detergents and eluted in 1 ml fractions with 0.1M glycine, PH 3.0, 0.5% Triton-X 100. The fractions were collected in tubes containing 60 µl 1M Tris pH 8.0.

Fractions containing radioactivity were pooled, made 10% in glycerol and 2% in SDS, heated at 60° C for 20 min, loaded onto a 10% preparative polyacrylamide gel in a single lane 10 cm wide, and electrophoresed for 16 h at 40 volts. A 0.5 cm wide strip of the gel was dried and autoradiographed and the rest stained with Coomassie blue. Stained bands containing T11 were localized by comparison with the migration of surface labelled T11 as shown by the autoradiographed strip. Three regions of the gel at approximately 55, 53 and 50KD were excised. Gel slices were washed with H<sub>2</sub>O and electroeluted in 50mM ammonium bicarbonate containing 0.1% SDS for 16 h at 50 volts (Hunkapiller et al., 1983). Sample eluates were collected and proteins precipitated at -20° C for 16 h by the addition of 9 volumes of cold ethanol. Precipitated proteins were collected by centrifugation and the protein pellets vacuum dried.

The ethanol precipitates were resuspended in 0.1% SDS and proteins were sequenced by Edman degradation on a gas phase protein sequenator (Applied Biosystems, model 120A) using the 03RPTH program and aqueous trifluoroacetic acid conversion chemistry. The PTH amino acids were identified with an on-line PTH analyzer (Applied Biosystems, model 120A) using a narrow-bore C18 reverse phase column run in acetate buffered 5% aqueous tetrahydrofuran and developed with acetonitrile. 5-10 µg from the 55, 53 and 50 kD bands were analyzed and an identical N-terminal sequence was obtained for each. 18 of the 19 N-terminal positions were assigned as follows:

55                    1                    10                    18  
K E I T N A L E T     ~~X X X~~     G A L G O D I N

Table 1

Cytolytic T-cell	Conditions	Anti-T11 <sub>2</sub> + Anti-T11 <sub>3</sub>		%Specific <sup>51</sup> Cr release	
				Laz 509	Laz 156
QQ	Control	-		6	81
QQ	Control	+		47	68
QQ	5 mM EGTA	-		0	1
QQ	5 mM EGTA	+		2	7
QQ	25°C	-		2	42
QQ	25°C	+		3	5
None	Supernatant*	-		1	0
QQ	Anti-T8	-		6	9
QQ	Anti-T8	+		50	54

\*Supernatant of clone QQ triggered with anti-T11<sub>2</sub> and anti-T11<sub>3</sub> at final dilutions of 1:100 each for 4 h.

Referring still to Table 1, the lysis of Laz 509 and Laz 156 by T-cell clone QQ was measured in the absence and presence of anti-T11 antibodies in the indicated conditions. Anti-T11 antibodies were used in ascites form at a 1:100 final dilution. In one set of wells, QQ cells were omitted and replaced with cell-free supernatants of clone QQ triggered with anti-T11<sub>2</sub> and anti-T11<sub>3</sub> for 4 h. The supernatants were used at a 50% final concentration.

Calcium is known to be required in the lethal hit stage of antigen-specific cytotoxicity. We found that it is also required for anti-T11-induced cytotoxicity because lysis is almost completely inhibited by EGTA. In addition, both antigen-specific and anti-T11-induced cytotoxicity are inhibited to some extent by low temperature; anti-T11 antibodies are almost completely inhibited at 25°C, whilst specific killing of Laz 156 by QQ is reduced by ~50%. No stable cytolytic factor was detected in the supernatants of T-cell clones triggered with anti-T11<sub>2</sub> plus anti-T11<sub>3</sub> in this assay system. Consequently, it is likely that either cell contact or close effector-target cell proximity is required for anti-T11-induced cytotoxicity.

These mechanistic similarities between antigen-specific and anti-T11-induced killing suggest that T-cells damage target cells in the same way whether activated through the T3 antigen receptor pathway or via the T11 molecule. Although T11 triggering induces lymphotoxin (LT) secretion by T-cell clones, it is unlikely that anti-T11-induced cytotoxicity is mediated by LT alone because the anti-T11-induced lytic effect can be measured in a 4-h assay using a variety of target cells, including those which are resistant to the effects of LT. In contrast, the detection of LT-mediated cytotoxicity generally requires sensitive target cell lines, longer assays and prior target cell conditioning.

The vast majority of human Natural killer (NK) cells, like early thymocytes, lack T11 α-chain messenger RNA, and consequently express no surface T3-T11 complex. On the other hand, the 50 kD T11 glycoprotein is found on the surface of most NK cells. These results suggest that certain NK cells may be related to T-lineage precursors.

Referring to Fig. 3, various target cells were incubated with T<sub>c</sub> and NK clones in the absence (a) or presence (b) of anti-T11<sub>2</sub> and anti-T11<sub>3</sub>. In the absence of monoclonal antibodies, the representative T11<sup>+</sup>T3<sup>+</sup>NK<sup>+</sup> clone JT3 efficiently kills the NK-sensitive target cell K562 but not peripheral blood lymphocytes of either of the two B-lymphoblastoid target lines tested. This specificity pattern is clearly distinct from that of the Laz 156-specific T-cell clone QQ. However, after incubation with anti-T11<sub>2</sub> and anti-T11<sub>3</sub> antibodies, both JT3 and QQ kill all of the target cells to varying degrees. Similar data were obtained for the independently derived NK cell clone JT816 (Hercend et al., 1983, 301 Nature 158). This ability of anti-T11 antibodies to induce killing activity from cells that lack a T3-T11 antigen/MHC receptor complex suggests that the T11 molecule represents an alternative pathway of cell activation. More importantly, these findings suggest that the T11 molecule is a critical structure for inducing cytolytic function in NK cells as well as T-cells.

Referring to Fig. 5, the 1.6Kb insert of PB1 (solid line) was separated from the plasmid by BamHI digestion and subcloned into the M13 sequencing vector mp18. The M13 universal primer was used to derive initial sequence at the 5' end. Subsequently, primers of 17 nucleotides were used for dideoxy sequencing. Sequencing reactions were also performed on M13 clones which contained BamH I-Sac I and Sac I-BamH I fragments as shown. The open reading frame of the insert is identified by the thick solid bar.

Referring to Fig. 6, there is shown the cDNA and predicted protein sequence of PB1. The probable signal peptide (—), the NH<sub>2</sub>-terminus of the mature protein (↘) and the position of a CNBr cleavage derived fragment (↗) are shown. Polyadenylation signals at nucleotides 1103 and 1505 are underlined. The last nucleotide before the poly(A) tail in clone PB2 is indicated by an arrowhead at position 1125.

The complete nucleotide sequence of clone PB1 is 1,522 bases in length and flanked by a poly(A) sequence at its 3' end. An open reading frame of 1,080 bases (positions 24-1103) begins with an ATG methionine codon and is flanked by 23 nucleotides of 5' untranslated sequence and by 419 bases of 3' untranslated sequence. A polyadenylation signal (AATAAA) is located 18 bases upstream from the beginning of the poly(A) tail (Fig. 6). PB2 is identical to PB1 except that it lacks four nucleotides present at the 5' end of PB1 and has a shorter 3' untranslated region. A poly(A) tail was noted in PB2 after the nucleotide corresponding to residue 1125 in PB1 (arrow head). The bases from positions 1102 to 1103 form part of a Gln codon at amino acid 336 and a stop codon as well as the first five bases of the polyadenylation signal AATAAA (nucleotides 1102-1106) for mRNA corresponding to clone PB2.

The N-terminal lysine of the mature polypeptide is preceded by a sequence coding for a stretch of 24 hydrophobic amino acids which likely represents the signal sequence required for the T11 precursor to be transported across the endoplasmic reticulum. The cDNA sequence predicts three potential N-linked glycosylation sites (Asn-X-Ser/Thr) on the mature protein at amino acid positions 65, 117 and 126. An extremely hydrophobic stretch of 25 amino acids characteristic in size and composition of a transmembrane domain is found at positions 186-210. This region is followed by seven basic amino acids within the next ten residues consistent with the notion that this is the start of the intracytoplasmic domain. 21% of residues in the region of amino acids 211-336 are prolines. This cDNA sequence predicts a molecular weight for the mature polypeptide backbone of 37,994 daltons.

To determine the molecular weight of the broad 50-55KD band in the absence of N-linked sugars, surface labelled T11 was digested with endoF and analyzed by SDS-PAGE. After digestion with endoF, there is a loss of the 55KD band and the appearance of a major component at approximately 40KD. These data suggest that the 55KD protein exists as a 40KD structure in the absence of any N-linked sugar moieties and is consistent with the above determined molecular weight of the protein. These results are consistent with the possibility that all three N-linked glycosylation sites may be utilized.

#### Two related T11 mRNA species

Northern analysis using polyA<sup>+</sup> RNA or cytoplasmic RNA from a variety of sources indicates that expression of the PB1 and PB2 sequences is T lineage specific and yields two common bands of 1.7 and 1.3Kb. Thus, each of six human T lineage cells tested including activated T helper clones, thymus derived tumors, normal thymocytes, activated T cells, and resting peripheral blood T lymphocytes expresses the 1.7 and 1.3Kb transcripts. In contrast, non-T lineage cells such as normal peripheral blood B cells and macrophages, an EBV-transformed B lymphoblastoid line, Laz 509, and the non-lymphoid hematopoietic cell lines HL-60 and U937 lack both transcripts. Given the similarity in size differences between the PB1 and PB2 inserts (~400 bases) and the two species of mRNA, it is likely that PB1 and PB2 cDNAs correspond to the 1.7Kb and 1.3Kb transcripts, respectively. This is further supported by the finding that an oligonucleotide based on a sequence from the 3' untranslated region unique to PB1 selectively hybridizes in northern analysis to sequences in the 1.7Kb site region.

Considerable variability exists within the level of expression of the 1.7 and 1.3Kb transcripts among individual T lineage tumor cells and physiologic T cell populations. Also, activated T cells express >10 fold higher amounts than resting T lymphocytes. This result is consistent with data indicating that T11 surface expression increases from 20,000 to 200,000 copies per cell upon activation with mitogens or antigens during a six day period and suggests that this differential expression is at least in part transcriptionally regulated.

Cells transfected with PB1, like T-cells which naturally produce T11, do not secrete T11, but rather retain the T11 molecule by means of the transmembrane anchor, with only the external domain exposed. A truncated T11 molecule, with the transmembrane and cytoplasmic domains deleted, can, unlike the complete T11 molecule, be secreted by transfected cells and used in the applications described below.

5 Such a truncated molecule can be prepared in a manner analogous to the method by which an anchor-minus IL-2 receptor molecule was prepared by Treiger et al. (1986) *J. Immunol.* **136**, 4099. The truncated IL-2 receptor was found to be capable of binding to its ligand, IL-2.

To make the truncated T11 molecule, PB1 or PB2 cDNA representing the gene for the entire T11 molecule will be restricted with Pvu II. This enzyme uniquely cuts within the 1,522 base pair T11 molecule cDNA insert at base 629, resulting in removal of all transmembrane and intracytoplasmic sequences and seven amino acids of the external domain. Subsequently, a 14 base phosphorylated synthetic oligonucleotide (CTAAGAATTCTTAG) containing the third base of the codon for amino acid 178, a termination codon TAA followed by the six base recognition sequence for EcoR I (GAATTC), and four nucleotides (TTAG) complementary to CTAA, will be ligated to the Pvu II restricted plasmid DNA with T4

15 DNA ligase. The DNA will then be digested with Pst I to separate the 5' end of the cDNA insert from the plasmid DNA and then subcloned into an appropriate expression vector. For example, this fragment could be blunted by T4 DNA polymerase to remove the Pst site and then be ligated to the EcoR I linker by T4 ligase. Finally, it could be digested with EcoR I before ligation into the unique EcoR I site of the publicly available PcEXV-1 expression vector.

20

### Use

Human NK and T<sub>c</sub> cells can be activated via the T11 molecule in vitro or in vivo and used in the treatment of any medical condition characterized by the presence of unwanted cells. In particular, the method can be used to turn on the cytotoxicity of T<sub>c</sub> and NK cells so that they will attack and kill pathogen-infected cells, e.g., cells infected with bacterial, fungal, viral, or protozoan pathogens; or tumor cells, e.g., lung, colorectal, or esophageal cancers.

The first step in in vitro activation is to obtain resting NK and/or T<sub>c</sub> cells, either from the patient or a suitable donor. This is typically done by separating out lymphocytes from blood and then, optionally, culturing the lymphocytes in the presence of Interleukin-2 to expand their numbers. (The methods by which lymphocytes are separated out, cultured, treated with Interleukin-2, and used to treat cancer patients are described in detail in Rosenberg et al. (1985) *New Eng. J. Med.* **313**, 1485.)

The lymphocytes and/or NK cells are incubated with the cytotoxicity-inducing substance for a relatively short time period (e.g., four hours at 30-40°C. in the presence of calcium ions), and subsequently infused into the patient. Infusion can be via an arterial or venous catheter or into a large peripheral vein.

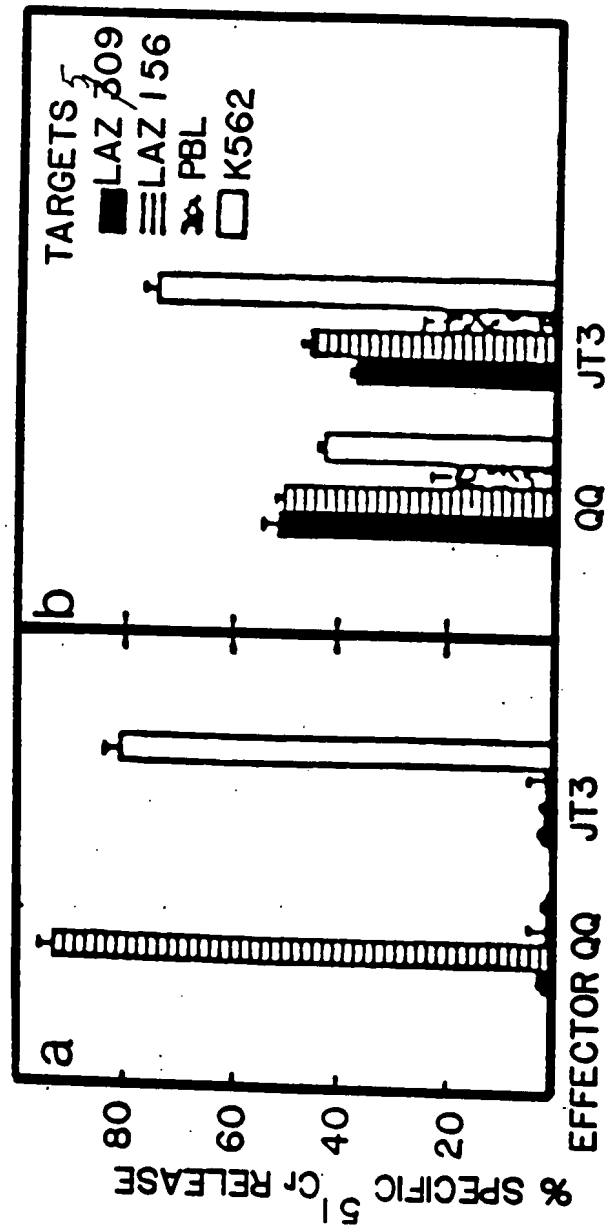
Alternatively, rather than activating T<sub>c</sub> and NK cells in vitro, the activating substance can be administered in vivo, most preferably by direct perfusion of the tumor with the substance, e.g., via the hepatic artery to induce cytotoxicity of existing NK and T<sub>c</sub> cells.

40 The methods of the invention induces cytotoxicity in a way which bypasses the normal T11 recognition mechanism, and enables the resultant activated NK and T<sub>c</sub> cells to attack and kill their target cells without further treatment. (NK cells have a broad range of target specificity, while T<sub>c</sub> cells recognize tumor cells by virtue of tumor-specific antigens.) Unlike treatment with IL-2 alone, treatment according to the invention will activate the totality of NK and T<sub>c</sub> cells, and be complete in a few hours.

45 The T11 protein, or its truncated, secreted form, can be used in a variety of diagnostic and therapeutic applications, all of which are based on the binding of T11 to its natural ligand on human lymphocytes and homologous surface structures present on target cells which facilitate T lymphocyte - target cell interactions, which result in target cell lysis or lymphocyte proliferation. (Siliciano et al. *Nature* **317**:428-431 (1985); and Palacios and Martinez-Maza. *J. Immunol.* **129**:2479-2485 (1982).)

50 The T11 molecule is expressed on the surface of many human T-cell malignancies, e.g., T-cell leukemias and lymphomas. In addition autoimmune diseases, e.g., rheumatoid arthritis and Systemic Lupus Erythematosus (SLE), are characterized by the presence in the blood and lymph of large numbers of T11-bearing T-cells. Rapid cell turnover in these disease states can cause the shedding of the T11 molecule into the bloodstream.

55



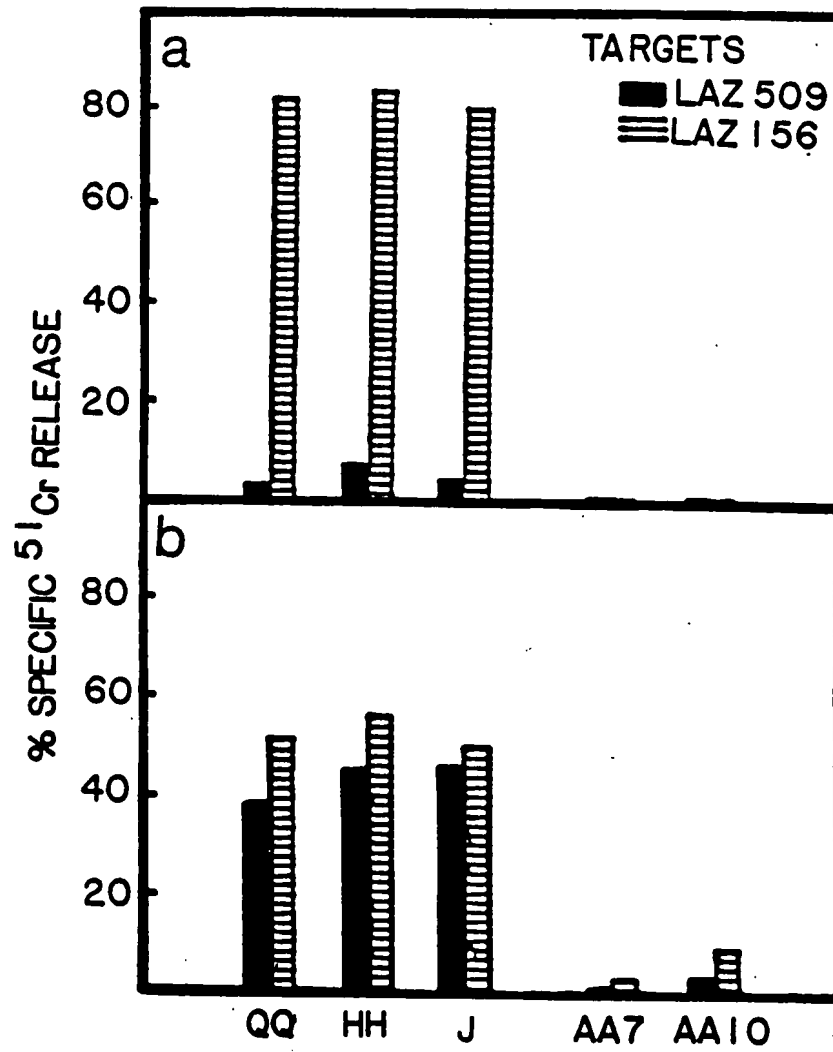


FIG. 2

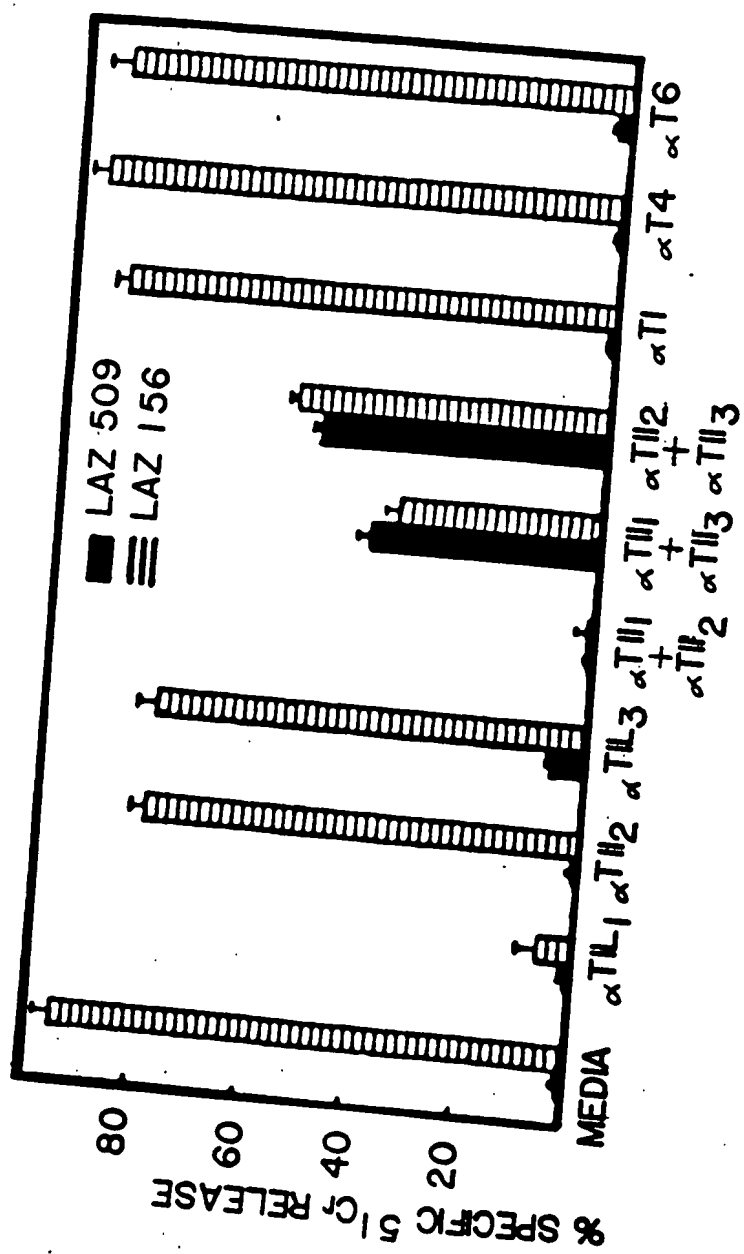


FIG. 3



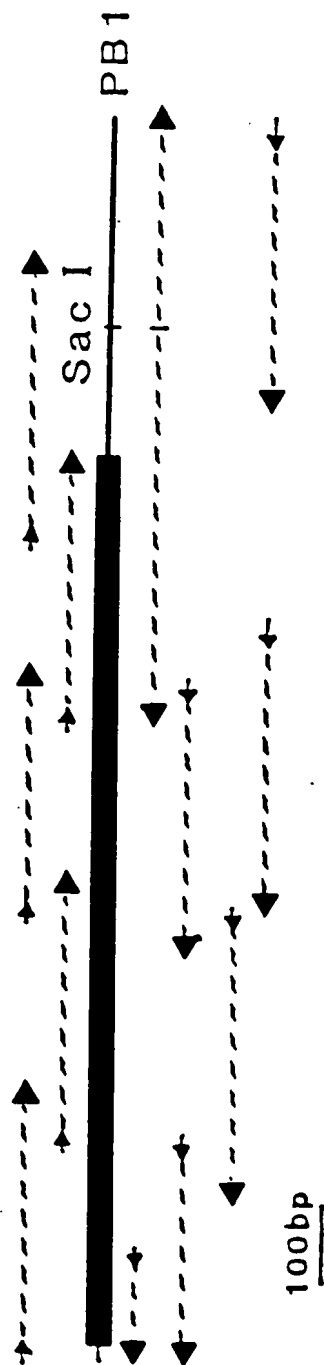


FIG. 4

10  
 AAAGAGGAA ACCAACCCCT AAG ATG AGC TTT CCA TGT AAA TTT GTA GCC ACC TTC 53  
 20  
 MET Ser Phe Pro Cys Lys Phe Val Ala Ser Phe -14  
 68  
 CTT CTG ATT TTC AAT GTT TCT TCC AAA GGT CCA GTC TCC AAA GAG ATT ACC AAT 98  
 Leu Leu Ile Phe Asn Val Ser Ser Lys Gly Ala Val Ser Lys Glu Ile Thr Asn  
 113  
 128  
 CCC TTG CAA ACC TGG GGT GCC TTG GGT CAG GAC ATC AAC TTC GAG ATT CCT AGT 158  
 Ala Leu Glu Thr Trp Gly Ala Leu Gly Gln Asp Ile Asn Leu Asp Ile Pro Ser 23  
 173  
 TTT CAA ATG AGT GAT GAT ATT GAC GAT ATA AAA TCG CAA AAA ACT TCA GAC AAG 218  
 Phe Gln MET Ser Asp Ile Asp Asp Ile Lys Trp Glu Lys Thr Ser Asp Lys 41  
 233  
 AAA AAG ATT GCA CAA TTC ACA AAA CAG AAA GAG ACT TTC AAG CAA AAA GAT ACA 263  
 Lys Lys Ile Ala Gln Phe Arg Lys Glu Lys Glu Thr Phe Lys Glu Lys Asp Thr 59  
 278  
 TAT AAG CTA TTT AAA AAT GCA ACT CTG AAA ATT AAG CAT CTG AAG ACC GAT CAT 323  
 Tyr Lys Leu Phe Lys Asn Gly Thr Leu Lys Ile Lys His Leu Lys Thr Asp Asp 77

FIG. 5-1

FIG. 5-2

338	CAC GAT ATC TAT AAG GTA TCA ATA TAT GAT ACA AAA GCA AAA AAT CTC TTC CAA	368	93
	Gln Asp Ile Tyr Lys Val Ser Ile Tyr Asp Thr Lys Gly Lys Asn Val Leu Glu		
383	398	413	428
AAA ATA TTT GAT TTG AAG ATT CAA GAG ACC CTC TCA AAA CCA AAG ATC TCC TCG			
Lys Ile Phe Asp Leu Lys Ile Gln Glu Arg Val Ser Lys Pro Lys Ile Ser Trp			113
443	458	473	488
ACT TGT ATC AAC ACA ACC CTC ACC TGT GAG GTA ATG AAT GCA ACT GAG CCC CAA			
Thr Cys Ile Asn Thr Thr Leu Thr Cys Glu Val MET Asn Gly Thr Asp Pro Glu			131
503	518	CNBr	533
TTA AAG CTG TAT CAA GAT GCG AAA CAT CTA AAA CTT TCT CAG AGG CTC ATC ACA			
Leu Asn Leu Tyr Tyr Gln Asp Gly Lys His Leu Lys Leu Ser Gln Arg Val Ile Thr			149
548	563	578	593
CAC AAG TGG ACC ACC AGC CTG AGT GCA AAA TTC AAG TCC ACA GCA GCG AAC AAA			
His Lys Trp Thr Thr Ser Leu Ser Ala Lys Phe Lys Cys Thr Ala Gly Asn Lys			167
608	623	638	
CTC ACC AAG GAA TCG AGT GTC GAG CCT GTC ACC TGT CCA GAG AAA GGT CTC GAC			
Val Ser Lys Glu Ser Ser Val Glu Pro Val Ser Cys Pro Glu Lys Gly Leu Asp			185
653	668	683	698
ATC TAT CTC ATC ATT GCG ATA TGT GCA GCA GCG ACC CTC TTC ATG CTC TTT CTC			
Ile Tyr Leu Ile Ile Gly Ile Cys Gly Gly Glu Ser Leu Leu MET Val Phe Val			203

FIG. 5-3

713	728	743	758
GCA CTC CTC GTT TTC TAT ATC ACC AAA AGG AAA AAA CAG AGC AGT CCG AGA AAT			
Ala Leu Leu Val Phe Tyr Ile Thr Lys Arg Lys Lys Gln Arg Ser Arg Arg Asn			
773	788	803	221
CAT GAG GAG CTC GAG ACA AGA GCG CAC AGA GTA GCT ACT GAA CAA ACC GCC CCG			
Asp Glu Glu Leu Glu Thr Arg Ala His Arg Val Ala Thr Glu Glu Arg Gly Arg			
818	833	848	239
AAG CCC CAC CAA ATT CCA GCT TCA ACC CCT CAG AAT CCA GCA ACT TCC CAA CAT			
Lys Pro His Gln Ile Pro Ala Ser Thr Pro Gln Asn Pro Ala Thr Ser Gln His			
878	893	908	257
CCT CCT CCA CCA CCT GGT CAT GGT TCC CAG GCA CCT AGT CAT CCT CCC CCG CGT			
Pro Pro Pro Pro Gly His Arg Ser Thr Pro Gln Ala Pro Ser His Arg Pro Pro Pro			
923	938	953	273
CCT GCA CAC GGT GTT CAG CAC CAG GGT CAG AAC AGC CCT GGT GGT CCC TCG CCG			
Pro Gly His Arg Val Gln His Gln Pro Gln Lys Arg Pro Pro Ala Pro Ser Gly			
983	998	1013	293
ACA CAA GTT CAC CAG CAG AAA GCG CCG CTC CCC AGA CCT GCA GTT CAG CCA			
Thr Gln Val His Gln Gln Lys Gly Pro Pro Leu Pro Arg Pro Arg Val Gln Pro			
1043	1058	1073	311
AAA CCT CCC ATC CCG CAG CAG AAA ACT CAT TGT CCC CTT CCT CTA ATT AAA AAA			
Lys Pro Pro MET Gly Gln Gln Lys Thr His Cys Pro Leu Pro Leu Ile Lys Lys			
			329

GAT AGA AAC TGT GTT TTT CAA TAA	1103	1116	1126	1136	1146
ASP ARG ASN CYS LEU PHE GLN					336
TACTTCCATG AGCTGTTTTC TGTCTCCAGA ACATTGTCAAC CTCTCGAGGC TGTGGGCCAC AGCCACCTCT	1136	1166	1176	1186	1196
					1206
GCATCTTCGA ACTCAGCCAT GTGGTCMACA TCTCGAGTTT TTGGTCTCTT CAGAGAGCTC CATCACACCA	1226	1236	1246	1256	1266
					1276
GTAMGCAGAA GCAATAAAG TGTGATTCCA AGAAGTGTAAG AGCACCAGGC CAGAAATCTT ACAGATTICT	1296	1306	1316	1326	1336
					1346
TGTCCTCTCT CAGGTCACTGT GTAGATGCCA TAAATCAAGT CATTTGGTGTG CCTGGCTCTC ACTACAAGCA	1366	1376	1386	1396	1406
					1416
CCCTATCTGC TTAAAGAGACT CTCGAGTTTC TTAATGTCCC TCGTGGACAC TTGCCACCA TCCTCTCAGT	1436	1446	1456	1466	1476
					1486
					1496

**FIG. 5-4**

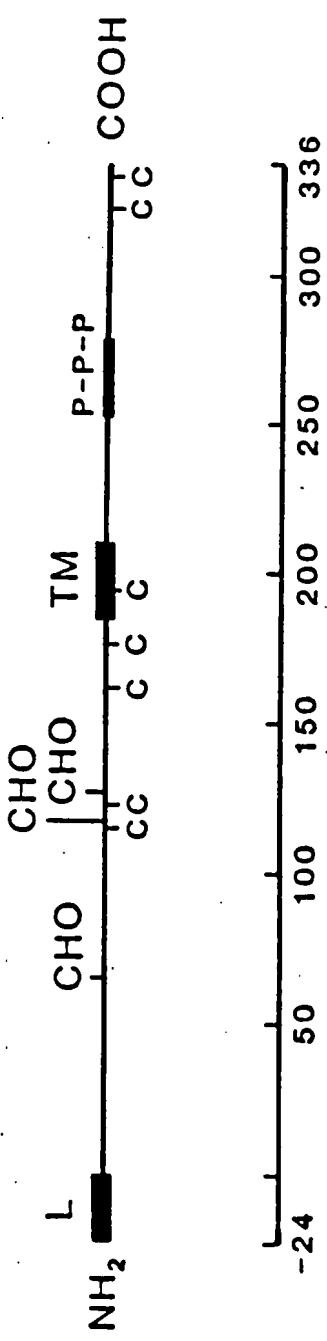


FIG. 6